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VOLUME IV NUMBER 4

STAIN TECHNOLOGY

OCTOBER, 1929

CONTENTS

Progress in the Standardization of Stains

Hematein and Acid Fuchsin. H. J. Conn 97

Stains recently certified 98

Professor P. G. Unna. Eugen Unna 100

The Variation in Staining Character of Bacteria as Related to the Reserve Food Material within the Organism. Esther W. Stearn and Allen E. Stearn 105

The Mechanism of Staining explained on a Chemical Basis. I. The Reaction between Dyes, Proteins, and Nucleic Acid. Allen E. Stearn and Esther W. Stearn 111

Staining of Negri Bodies. J. H. Jordan and A. H. Heather 121

A Modification of Mayer's hemalum. John E. Sass 127

A Short Method of Clearing Plant Tissues for Anatomical Studies. Jennie L. S. Simpson 131

Laboratory Hints from the Literature 133

Contains Title Page, Table of Contents and Index to Vol. IV

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Address all correspondence regarding manuscripts for publication, proofs, etc. to H. J. Conn, Lock Box 299, Geneva, N. Y. Articles over two printed pages in length (i. e. about 800 words) must be preceded by a brief summary (100–300 words) which can be used as an author's abstract by abstracting journals. Authors are especially urged to make all staining formulae definite and accurate. If known to the author, the dye content of any stain should be given and the exact dye used should be specified.

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STAIN TECHNOLOGY

VOLUME IV OCTOBER, 1929 NUMBER 4

PROGRESS IN THE STANDARDIZATION OF STAINS

HEMATEIN AND ACID FUCHSIN

The Commission must acknowledge the assistance recently given by two commercial concerns in developing two new products, namely, a pure type of hematein and a special grade of acid fuchsin.

Hematein is well known as an oxidation product of hematoxylin. The hematein at present on the market, whether domestic or imported, seems to be prepared from crude hematoxylin and is quite unsatisfactory. A new product has just been developed, however, by MacAndrews & Forbes of Camden, N. J., which is derived from pure hematoxylin and has been found to be a splendid stain. This new product will soon be put on the market under Commission certification and will be available not only from the company just named, but from the regular dealers in biological stains. The advantage of hematein over hematoxylin itself is that staining solutions containing it can be used immediately without waiting for them to ripen.

A new type of acid fuchsin has recently been developed after considerable experimental work by the National Aniline & Chemical Co., according to specifications given by Scanlan, French and Holmes. This type of acid fuchsin seems to be much more satisfactory for all the purposes for which this dye is employed than the type previously on the market. At the present time only one batch of this new type of acid fuchsin has been submitted to us for certification. This type is to be sold under the certification number NR-6. It is planned in the future to approve only this type of acid fuchsin, but there are still acid fuchsins of the older type on the market and undoubtedly will be for some time to come. These latter are entirely satisfactory for most procedures, but in some special work the new type is much more desirable.

H. J. CONN

STAIN TECHNOLOGY, Vol. 4, No. 4, Oct. 1929

Stains Recently Certified

In the table below is given a list of the batches of stain approved since the last one listed in the July number of this journal.

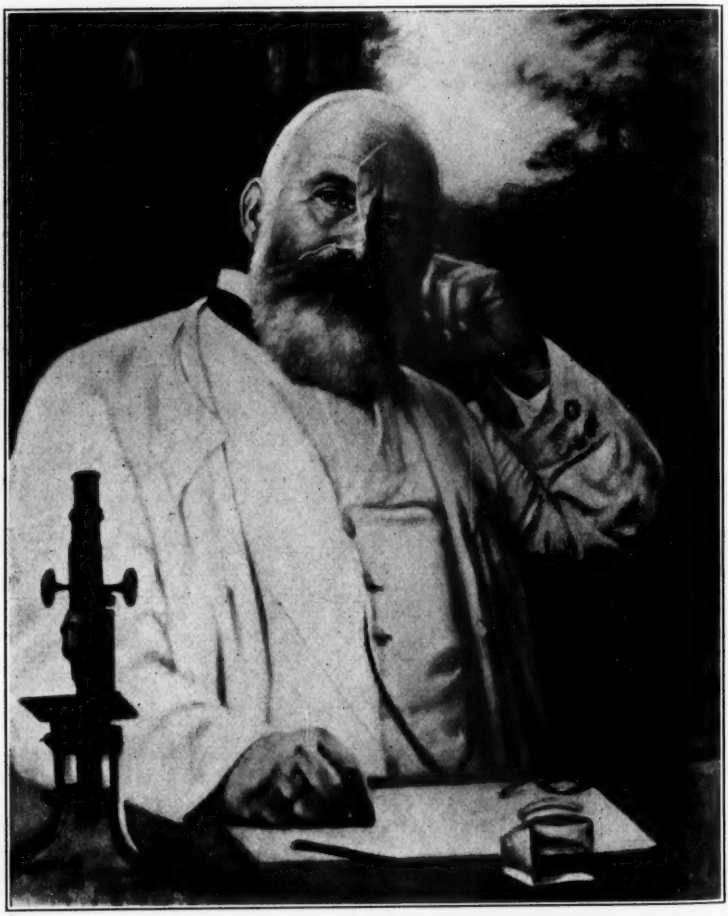
Stains Certified May 15 to August 15, 1929*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Fuchsin, basic	CF 9	91%	As bacterial stain; not satisfactory in Endo medium	June 5, 1929
Fuchsin, basic	NF 15	85%	For staining and for use in Endo medium	June 5, 1929
Fuchsin, acid	NR 5‡	62%	As histological stain and for use in Andrade indicator	June 5, 1929
Crystal violet	NC 8	91%	As bacteriological and histological stain	June 5, 1929
Eosin Y	CE 4	87%	As histological stain	June 5, 1929
Phloxine	CPh 2	88%	As histological stain	June 5, 1929
Azure A	NAz 3	——	As histological stain	June 5, 1929
Methyl orange	NM 2	96%	As histological stain	June 25, 1929
Methylene blue	CA 9	87%	As bacteriological and histological stain	July 23, 1929
Bismarck brown Y	CN 2	67%	As histological stain	July 29, 1929
Methyl green	CG 2	49%	For histological use and as constituent of Pappenheim stain	July 29, 1929
Erythrosin	CEr 1	83%	As histological stain	Aug. 5, 1929

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

‡Certified provisionally pending investigations by the company as to the manufacture of acid fuchsin by the methods of Scanlan, French, and Holmes.



DR. P. G. UNNA
1850–1929

PROFESSOR P. G. UNNA*

EUGEN UNNA, *Hamburg, Germany*

Professor P. G. UNNA died of influenza in Hamburg on January 29th of this year, at the age of seventy-nine.

Born in Hamburg in 1850, Prof. Unna came of a family which had for generations been connected with the medical profession. In 1870, while still a medical student at Heidelberg, he served as a volunteer in the Franco-Prussian War and was badly wounded in the fighting around Le Mans. He obtained the state diploma at Strassburg where he studied under such men as von Recklinghausen, Hoppe-Seyler, Schmiedeberg, amd Waldeyer. In 1876 he went for a short time to Vienna where he first met Hebra, the founder of modern dermatology. From 1876 to 1878 he acted as assistant to Engel-Reimers at the St. George's Hospital, Hamburg; in 1881 he opened his private clinic and the following year he, together with Oscar Lassar and Hans Hebra, founded the "Monatshefte fur praktische Dermatologie," now known as the "Dermatologische Wochenschrift." In the years 1887–88 he visited England, France, Scandinavia, and U. S. A. From 1888 onwards scientific courses have been held at the "Dermatologicum;" in 1907 he was nominated professor by the senate of his native city; in the following year he was appointed senior physician to the hospital at Eppendorf and in 1919 was elected to the Chair of Dermatology at the new University of Hamburg. In 1927 he received the degree of Dr. Phil. h.c. of the philosophical faculty of the University of Bonn, and he was an honorary member of numerous scientific societies. That, in outline, is the record of a life which was singularly harmonious alike in its inner and its outer development.

Prof. Unna's M.D. thesis on "Skin Development" (1875) revealed the basis of his method of research both in the choice of the subject and in its treatment; already an original and ingenious method of staining opened out to him a new vista of facts and perceptions. Even at that stage of his career he sought to obtain chemical and biological results from the different way in which the tissues react to staining. This was a favorite idea of his, he always kept at it, and it permeated the work of a life-time.

He wrote more than a thousand articles and essays, but it is impossible even to refer to them within the limits of this brief obituary

*The many contributions to staining, made by the late Dr. P. G. Unna make his life of much interest to readers of this journal. We are very fortunate in securing Dr. Eugen Unna to write specially for us this account of his father's contributions.—ED.

notice.—As an illustration I may recall his "Histopathology of Diseases of the Skin," a treatise of 1225 pages which is still today the standard work on dermatology and is likely to remain so for generations to come. Mention may also be made of his discovery of the streptobacillus of soft chancre, as well as his methods for presenting the parasites of the stratum corneum, which alone entailed a labor of 3000 experiments.—It will also be remembered that seborrhoic eczema, a well-known clinical entity, is always associated with the name of Unna as well as the terms elacin and plasma cells.

The teachings on which he laid the greatest stress and to which he was undoubtedly justified in attributing a general physiological and biological value, are those referring to the areas of oxidation and reduction ("Sauerstofforte, Reductionsorte"). This conception is the outcome of a long line of investigations during the years 1906–1915, based on tests in which living and surviving tissues are treated with reagents (potassium permanganate solution and reduced methylene blue, rongalit white). His most remarkable achievement is the discovery of plasma cells. They are stained with polychrome methylene blue, and subsequently decolorized with glycerine-ether, or with Pappenheim-Unna's methyl-green-pyronin and phenol; they are distinguished by an excessive amount of granular, acid, oxidizing material, called granoplasm, which is superimposed to the basic, reducing spongioplasm. Granoplasm is a secondary albumose. Unna succeeded in isolating it in quantities and termed it cytose.

Mast cells are connective tissue cells, containing strong oxidizing and strong acid granules that stain red with polychrome methlyene blue, i.e. they are metachromatic.

The technical methods with which Unna succeeded in establishing these micro-chemical results, form part of a system which he later on called *Chromolysis*. It investigates the constitution of tissue structures under certain stains by which they are defined, and under different solvents which make them disappear again.

In dermatology, Unna achieved much wider fame both in his own country and thruout the world. It is well known that he is the father of seborrhoic eczema, and also of the group of Ulerythemas, that he likewise described the pathology of senile purpura; that he discovered a special form of vesicles called by him balloon-like degeneration ("ballonnierende Degeneration"), a characteristic feature of chicken-pox and herpes zoster; that he, again, proved the epithelial origin of naevi and naevo-carcinoma, and showed that urticaria pigmentosa is caused by an accumulation of mast cells; that he also proved without doubt that *Bacillus Unna-Dukrey* is the specific organism in soft chancre; that he gave a complete definition

of impetigo and a remarkable description of impetigo staphylogenes; and that, finally, he cleared up the confusion in the questions relating to acne and proved that rosacea is entirely an affection of another kind.

His teaching on histological technic is less well-known than his anatomical writings, yet it was a field in which he was a creative genius and an acknowledged expert. He has tried more than once and especially during the latter years of his life to infuse into his essentially important works those principles that he wished to impart to others. To these important works belong his *Chromolysis, Areas of Oxidation and Reduction* in the Manual of Practical Biology (1921), his book on "*Biological Phenomena in the Skin of Man and Animals*" in conjunction with J. Schumacher (1925), and his last work "*Histochemistry of the Skin*" (1928), the latter however an abstract without the admirable colored plates. He might easily have decided to write a summary of his technical methods; that he did not do so may be attributed to the fact that he was perpetually trying to make further improvements. Now that he has passed away it might be appropriate in conclusion of this memoir to quote the tribute of Professor Dr. J. Darier to his friend Unna from the "Annals of Dermatology."

"Unna has bequeathed to us an enormous number of facts from which one ought to extract the gems or, at any rate, the guiding principles which deserve to be brought to light and carefully preserved. My special reason for saying this is to induce our younger colleagues to profit by the immense treasury of knowledge which this incomparable pioneer of science has accumulated. The labor of classifying and eliminating will certainly bear fruit. When faced with histological problems of any description or—to give a concrete example—with a puzzling tumor, e.g. a sarcoma, one would like to know of the correct working method of Unna's to be adopted for fixing, embedding, staining, and solution, the usual way of approaching the subject and the probable answer to the problem.

"By collecting the results in any branch of science we lay a safe foundation to further progress. Unna has done so much in the wide fields of his research that the time has come for us to start making an inventory before we turn to fresh investigations. The compilation of his scattered works which I am advocating would be a fitting way of honoring the memory of a great pioneer by whose passing science has suffered a very deep and grievous loss."

THE VARIATION IN STAINING CHARACTER OF BACTERIA AS RELATED TO THE RESERVE FOOD MATERIAL WITHIN THE ORGANISM

ESTHER WAGNER STEARN and ALLEN E. STEARN, (*Dept. of Preventive Medicine, and Div. of Physical Chemistry, Univ. of Missouri.*)

ABSTRACT—**When *Bacillus cereus* is starved it tends to lose Gram-positivity at 37°C., tho not at ice box temperature. In the latter case the cells are probably dormant undergoing no significant change, but the results in the former case are thought to be due to the using up of acidic reserve food materials (nucleins, nucleo-proteins, etc.) when the organism grows in the absence of a nutritive environment.**

Most studies concerning Gram-variability of bacteria have been concerned with conditions that bring about loss of dye retention and not with the actual changes in the structure of the organism itself. One of the most interesting studies of the latter type is that by Churchman[1]. He found that the addition of small amounts of aqueous gentian violet, acriflavine or acriviolet to suspensions of young cultures of *B. anthracis* not only reverses their Gram reaction but also causes a diminution in diameter of the cells of around 40%, and a loss in weight of the cells. The material removed from the cells by this treatment was found to give a positive nin-hydrin reaction. These facts he explains tentatively by assuming a Gram-positive cortex and a Gram-negative medulla, tho, as he says, "positive proof of the correctness of this explanation must await the evidence furnished by cross sections of bacteria." Similar changes were produced in many, but not in all, of the sporogenic aerobes, and the spores, when present, are entirely contained within the Gram-negative forms. These dyes, however, were without effect on the Gram-positivity of the majority of non-spore-formers. According to his findings there are two types of organisms, one which exhibits reversibility of the Gram reaction when exposed to the dyes, the other which does not.

In commenting on this Churchman says, "Since a certain amount of reversal occurs sooner or later in the control tubes to which no dye has been added, it is not unlikely that the dye simply promotes or hastens a change in the bacteria caused by degeneration or lysis (dependent perhaps on change of reaction), which would occur without any dye." His weighing experiments were performed with desiccated specimens, so that the actual loss in weight upon treatment accounts for the change in size.

[1]Churchman, J. W. The Structure of *B. anthracis* and Reversal of the Gram Reaction. J. Exp. Med., 46, 1007. 1927.

Certain results of Hucker and Conn are of interest in this general connection.[2] Gram-positive cocci, mostly isolated from pathogenic lesions and dairy products became doubtful or Gram-negative when over three days old. Of 200 cultures of non-spore-forming rods isolated from either soil or manure, 21 were found to vary from day to day in Gram reaction. Smears from the same agar slants of these latter cultures on the fourth and seventh day gave the greatest number of positive reactions. Only two cultures (both manure) were positive on either the first or second days, and negative on the fourth and seventh days.

The question arises (for the present authors) whether the first class, which loses its Gram-character as it ages, has been forced to acclimatize itself to a lessened nutritive environment, being forced to change from an environment (rich in animal proteins and fats) in which it could readily assimilate nutritive substances and in which prolonged storage of food in the cell was unnecessary, to one where the supply was gradually diminished. The latter class, however, changed its environment in the opposite direction, i.e. from an inorganic to a complex organic environment.

The storage of food substances, their use by the cell, and the subsequent gradual starvation of the organism must depend on the past environment and on the ability of each single cell to acclimatize itself to decreased or increased nutritive conditions.

As early as 1892 this factor was recognized by Mills[3] who found that Gram-positive bacilli from pus became Gram-negative when cultured on media. Many such cases can be cited but, so far as the authors are aware, no one has mentioned a case where a Gram-positive organism has become negative under conditions of change from a less to a more highly nutritive, complex, organic, ideal environment. Gram-negative organisms, however, have been shown to acquire positiveness at times when changed from a less to a more nutritive environment.

It would seem of interest to point out any correlation between change in Gram-character and vital activity, especially the ability of an organism to store reserve food material. That this stored reserve food material is an important factor in the staining properties of an organism can be shown. Thus Enderlein states[4] that the nuclear body is observable only when the cell holds but little food substance in reserve. This food reserve exists for the most part as ultramicro-

[2]Hucker, G. J. and Conn, H. J. Further Studies on the Methods of Gram Staining. N. Y. Agr. Exp. Sta., Tech. Bul., 128. 1927.

[3]Mills, A. Meningite a pneumocoques. J. Soc. Roy. d. Sci. Med. et Natur. Bruxelles, 1892, No. 29, 67. (Original not seen; cited from Baumgarten's Jahrsb. 8, 1892.

[4]Enderlein, G. Bakterien Cyclogenie. W. de Gruyter & Co., Berlin, 1925.

scopic granules, called trophoconia and represents the actual chromatic material of the cell. It stains strongly with basic dye due to its high content of nucleic acid and nucleo-proteins. When the reserve food in a cell has been used up, as in bacteria starved by keeping in distilled water, the last remnant of food reserve clings tenaciously about the nuclear body. Alcohol extraction is another method of removing such material from cells.

Schumacher[5] demonstrates this same point in his work on yeasts. Frozen sections 5 μ thick were stained. The yeast cells which were cut became Gram-negative, the cell content being released and going into solution. This released cell content can be precipitated out of solution and, when stained, will retain methylene blue, which he attributes to nucleo-protein, and rosanilin, which he attributes to lipo-protein.

Henrici has shown in the general case of living protoplasm that the Gram staining character is not uniformly distributed thruout the protoplasm.[6] Is this difference possibly dependent on the ability of the Gram-positive cells such as yeasts and some of the "larger" bacteria to store a certain kind of reserve food material? Henrici has pointed out that in animal tissue the nucleus retains the stain longer than the cytoplasm, but that all tissue elements are eventually decolorized with the exception of keratohyalin.

Benians found[7] that bacterial detritus retained a faint purplish color after five minutes' treatment with alcohol, while there is no color when Gram-negative bacteria have been stained in the same way and decolorized by alcohol. He claims that the Gram reaction is dependent on the physical attributes of the intact bacterial cell, yet he found that crushed yeast cells held the stain more strongly than did crushed sarcinae, that granules in yeast cells resisted decolorization longer than did the main portions of the cells, that *B. subtilis* showed a very marked granular appearance in the earlier stages of decolorization.

Experimental

A suspension of *Bacillus cereus* Frankland in physiological salt solution was incubated at 37°C for 48 hours. These organisms were then stained on slides on which were also smears of 24-hour cultures

[5]Schumacher, J. Zur Gramschen Färbung. Hat das der Grampositivität zugrundeliegende Lipoproteid der Hefezelle seinen Sitz in der Zell membran oder in Protoplasma? Centbl. f. Bakt., I Abt., Orig., **98**, 104. 1926.

[6]Henrici, A. T. The Staining of Yeasts by Gram's Method. J. Med. Res., **30**, 409. 1914.

[7]Benians, T. H. C. Observations on the Gram Positive and Acid Fast Properties of Bacteria. J. Path. and Bact., **17**, 199. 1913.

of *B. subtilis* Cohn and of a strain of the colon organism obtained from the Public Health Laboratory of the Univ. of Missouri. (The cultures of *B. cereus* and *B. subtilis* had been isolated by single cell technic from cultures obtained from the collection of the Society of Amer. Bacteriologists while located at Washington, D. C.) The *B. cereus* cells were found to be of a great variety—a few Gram-positive cells, some full size Gram-negative cells, Gram-negative vacuolated cells, certain Gram-negative cells of about half the ordinary diameter, similar to those described by Churchman (see footnote 1), and finally some Gram-negative cells containing Gram-positive granules. The suspension was subcultured to neutral nutrient agar slants. After periods of 24 and 48 hours this culture consisted predominantly of Gram-negative organisms. From these tubes the culture was suspended in sterile physiological salt and incubated for seven weeks at 37°C. The suspension, during this period, was stained at various intervals and subcultured, but the results at the end of the seven week period were the same as at the end of one week.

The suspension showed Gram-negative organisms, which not only did not retain the primary stain but also were only faintly stained by the counterstain (safranin) or by a stronger solution of basic fuchsin. Dorner's spore stain showed but few spores.

A 48-hour agar culture from this suspension showed about 40% of the organisms totally Gram-negative, and the rest stippled thruout with Gram-positive granules. None of the *B. cereus* cells showed homogeneous staining, tho the *B. subtilis* cells from a 24-hour culture on the same slide were solidly and homogeneously Gram-positive and the *B. coli* were similarly Gram-negative. These smears were repeatedly stained using not only the classic technic but also the modifications of Burke, of Hucker and of others. They were also stained by more than one person.

If the culture of *B. cereus* in the physiological salt be maintained at ice-box temperature in place of incubation temperature it will not change its Gram-character. This is explained by assuming that at 37°C the organism multiplies, using its reserve food material, while at ice box temperature the organism, when it does not sporulate, simply remains dormant.[8]

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[8]If, in the case of the above incubated suspension of *B. cereus*, a staining technic was employed using as a mordant a 0.5% solution of potassium dichromate, the organisms were stained a violet color. This is in accord with the theory of the authors as to the functions of such mordants. See Stearn, E. W. and Stearn, A. E. The Chemical Mechanism of Bacterial Behavior. I. Behavior toward Dyes—Factors Controlling the Gram Reaction. J. Bact., 9, 463. 1924.

Discussion

These results, on the whole, indicate that Gram positivity is due to a more or less specific portion of the cell content, but the indications are not in accord with the tentative suggestion of Churchman (see footnote 1) of a Gram-positive cortex and a Gram-negative medulla. The fact of many Gram-negative cells of full size militates against the hypothesis of a peeling off of a Gram-positive cortex, while the stippled appearance of the "partly starved" cells suggests the more or less uniform distribution of the material causing dye retention. These authors feel that the results of Churchman are as well explained by this hypothesis as by the one he suggests, since even the extraction of a considerable portion of cell content which is uniformly distributed would cause reduction in volume as well as in weight in cases of fairly high positive interfacial tension between cell and medium.

THE MECHANISM OF STAINING EXPLAINED ON A CHEMICAL BASIS. I. THE REACTION BETWEEN DYES, PROTEINS AND NUCLEIC ACID

ALLEN E. STEARN and ESTHER WAGNER STEARN, *Dept. of Bacteriology and Patten Research Laboratories, Northwestern Univ. Medical School, Chicago, Ill.*[1]

ABSTRACT—**Basic dyes cause an increase in hydrogen-ion concentration when added to a solution containing nucleic acid, tho both solutions were originally at the same pH. Acid dyes have no effect on nucleic acid solutions. Basic dyes show the same behavior when treated with solutions of typical proteins. Acid dyes when treated with proteins show an analogous effect but in the opposite direction. The only adequate explanation found is that there is a definite reaction between the dye ions and the oppositely charged ions of protein or nucleic acid. The bearing of these results on the theory of staining is discussed. The growing recognition of the dominance of chemical forces in colloidal adsorption behavior is indicated, and certain of the experimental bases for this recognition are pointed out and discussed.**

It is extremely difficult to obtain experimental evidence which will conclusively prove one type of staining mechanism and disprove others. Ordinary dyeing reactions have been explained as being due either to 1) chemical reaction between dye and material dyed, 2) distribution of dye between bath and material dyed, or 3) adsorption of dye by material dyed. In the last named case the forces acting may be either chemical or physical. Since the term adsorption carries this broad meaning there should be no lack of agreement that dyeing and staining reactions have an adsorption mechanism, if one excludes the very exceptional cases of distribution. The beauty of the generality of the adsorption idea is, however, more than offset, it has always seemed to the authors, by the fact that it explains very little if anything of mechanism. It constitutes in most cases merely a more elusive term for stating an empirically observed fact.

In the present paper data is presented which indicates very strongly that a definite chemical reaction is involved when dyes and proteins are brought together, or when certain classes of dyes and nucleic acid act on each other. The observations are, basically, the following. When a solution of, for example, gelatin or nucleic acid, adjusted to a

[1]Work performed while authors were summer visitors in these laboratories, respectively.

certain pH, is mixed with one of basic dye at the same pH there is, thru wide pH limits, a significant increase in hydrogen-ion concentration.

The only adequate explanation which the authors can offer is as follows. Consider the type system nucleic acid and crystal violet. The former, represented by the type formula HNu will ionize to furnish nucleate ions:

$$HNu \rightleftharpoons H^+ + Nu^- \qquad (1)$$

This ionization can be increased by any reagent which removes either H^+ or Nu^-. The first can be accomplished by neutralizing with a base. Data here presented indicate that the second can be accomplished by "neutralizing" the Nu^- with certain basic ions, represented by the general symbol D^+.

Thus $$Nu^- + D^+ \longrightarrow DNu \qquad (2)$$

This removal of Nu^- will then cause a shift in the equilibrium represented by equation (1) with corresponding increase in hydrogen-ion concentration.

If this mechanism is correct the following qualitative predictions should be found to be true:

1. Basic dyes should cause an increase in hydrogen-ion concentration when added to a solution containing some un-ionized nucleic acid.

2. Basic dyes should tend to lose this effect as the nucleic acid is transformed to sodium nucleate.

3. Acid dyes should not exhibit any effect of the same order of magnitude with nucleic acid.

4. Basic dyes should cause the same type of effect with the ampholyte, gelatin, as with nucleic acid.

5. Acid dyes may be expected to produce an analogous effect with the ampholyte gelatin, but it should be in the opposite direction; i.e. there should be a *decrease* in hydrogen-ion concentration.

How well these predictions are in accord with the experiments may be judged by examination of the data in Tables 1–4. The experiments are qualitative in nature and very easy to perform. The gelatin solutions were 1% in concentration and the nucleate solutions were 0.5%. The dye solutions contained 3 grams per liter. The first two named solutions are fairly well buffered and adjustment to a particular pH is not difficult. The dyes are not well buffered thru the range of solubility and adjustment is difficult. Where the two solutions are not at the same pH it will be seen that the dye will be in general at a pH *higher* than that of the other solution in case a

TABLE 1. NUCLEIC ACID WITH BASIC DYES.

Showing the increase in hydrogen-ion concentration, $[\Delta(H^+)]$, and the corresponding decrease in pH, $[-\Delta pH]$, when solutions of nucleic acid are mixed with those of basic dyes whose hydrogen-ion concentrations are equal to (or lower than) those of the nucleic acid solutions. These values are calculated in all cases from the pH values of the mixtures and of the nucleic acid solutions, since these latter solutions show greater buffer value than the plain dye solutions.

	Crystal Violet			Malachite Green			Brilliant Green		
	pH	$-\Delta pH$	$\Delta(H^+)$	pH	$-\Delta pH$	$\Delta(H^+)$	pH	$-\Delta pH$	$\Delta(H^+)$
1. dye.....	3.9			3.10			3.15		
2. acid.....	3.84			3.10			3.15		
3. mixture....	3.42	0.42	1.4×10^{-4}	3.02	0.08	1.6×10^{-4}	3.08	0.07	1.2×10^{-4}
1. dye.....	4.90			4.03			4.00		
2. acid.....	4.60			4.03			3.98		
3. mixture....	4.24	0.36	3.3×10^{-5}	3.84	0.19	5.2×10^{-5}	3.88	0.10	2.7×10^{-5}
1. dye.....	5.7			4.66			Brilliant green precipitated at these higher pH-values.		
2. acid.....	5.62			4.65					
3. mixture....	5.25	0.37	3.2×10^{-6}	4.40	0.25	1.8×10^{-5}			
1. dye.....	7.05			5.65					
2. acid.....	7.08			5.56					
3. mixture....	6.95	0.13	3.0×10^{-8}	5.36	0.20	1.6×10^{-6}			

TABLE 2. NUCLEIC ACID WITH ACID DYES.

Showing change in hydrogen-ion concentration when solutions of nucleic acid are mixed with those of acid dyes. (Here no appreciable change is noted.)

	Acid Fuchsin			Eosin		
	pH	ΔpH	$\Delta(H^+)$	pH	ΔpH	$\Delta(H^+)$
1. dye.....	3.08			3.97		
2. acid.....	3.08			3.97		
3. mixture.....	3.08	—	—	3.96	0.01	—
1. dye.....	3.94			4.65		
2. acid.....	3.96			4.66		
3. mixture.....	3.95	0.01	—	4.66	—	—
1. dye.....	4.67			5.54		
2. acid.....	4.65			5.56		
3. mixture.....	4.59	0.06	?	5.56	—	—
1. dye.....	5.60					
2. acid.....	5.57					
3. mixture.....	5.57	—	—			

pH *decrease* is obtained and vice versa thus eliminating any diluting effects. The pH of each solution was taken and then equal volumes were mixed and the pH of the resulting mixture determined. This often does not give the maximum effect, since there is no exact means of estimating stoichiometrical equivalents of ions in the various solutions. In most cases relative volumes can be found which, if mixed, will give an effect greater than that noted.

TABLE 3. GELATIN WITH BASIC DYES

Showing the increase in hydrogen-ion concentration, [$\Delta(H^+)$], and the corresponding decrease in pH, [$-\Delta pH$], when solutions of gelatin are mixed with those of basic dyes whose hydrogen-ion concentrations are equal to (or lower than) those of the gelatin solutions. These values are calculated from those of the mixtures and of the original gelatin solutions, which latter have a buffer value greater than the dye solutions.

	Crystal Violet			Malachite Green			Brilliant Green		
	pH	$-\Delta pH$	$\Delta(H^+)$	pH	$-\Delta pH$	$\Delta(H^+)$	pH	$-\Delta pH$	$\Delta(H^+)$
1. dye.....	3.92			3.02			3.12		
2. gelatin.....	3.91			3.00			3.12		
3. mixture....	3.83	0.08	2.5×10^{-5}	3.00	—	—	3.13	—	—
1. dye.....	4.64			3.95			Brilliant green insoluble at these higher pH values.		
2. gelatin.....	4.64			3.98					
3. mixture....	4.29	0.35	2.8×10^{-5}	3.90	0.08	2.0×10^{-5}			
1. dye.....	5.64			4.61					
2. gelatin.....	5.64			4.62					
3. mixture....	5.32	0.32	2.5×10^{-6}	4.46	0.16	1.05×10^{-5}			
1. dye.....	6.27			5.60					
2. gelatin.....	6.46			5.60					
3. mixture....	5.94	0.52	8.0×10^{-7}	5.25	0.35	3.2×10^{-6}			

NOTE.—A pH of 3 seems a little too far on the acid side of the isoelectric point for much effect of these basic dyes with gelatin.

The calculated change in hydrogen-ion concentration is based always on the original hydrogen-ion concentration of the buffered solution, i.e. the nucleic acid or the gelatin. A quinhydrone electrode was used and its potential measured by means of a potentiometer against a saturated calomel electrode.

There are two or three considerations besides the actual data which point to a chemical explanation. In the first place these effects are independent of whether or not precipitation of the dye nucleate or gelatinate takes place. In the second place the same kind of results

are obtained whether the protein is in solution or not. This type of experiment can be made on proteins since the dye solution can be adjusted to the isoelectric pH of the protein. In Table 5 a few such

TABLE 4. GELATIN WITH ACID DYES

Showing the decrease in hydrogen-ion concentration, $[-\Delta(H^+)]$, and the corresponding increase in pH, [ΔpH], when gelatin solutions are mixed with acid dye solutions whose hydrogen-ion concentrations are equal to (or greater than) those of the gelatin solutions.

	Acid Fuchsin			Eosin		
	pH	ΔpH	$-\Delta(H^+)$	pH	ΔpH	$-\Delta(H^+)$
1. dye	3.13					
2. gelatin	3.13					
3. mixture	3.32	0.19	2.6×10^{-4}	Eosin not soluble at these lower pH values.		
1. dye	3.75					
2. gelatin	3.75					
3. mixture	4.10	0.35	1.0×10^{-4}			
1. dye	4.60			4.58		
2. gelatin	4.62			4.62		
3. mixture	4.73	0.11	5.0×10^{-6}	4.70	0.08	4.0×10^{-6}
1. dye	5.64			5.61		
2. gelatin	5.62			5.61		
3. mixture	6.05	0.43	1.5×10^{-6}	5.68	0.07	3.5×10^{-7}

TABLE 5

Showing the decrease in pH, [−ΔpH], when crystal violet solutions are added to various ampholytes. The casein and gelatin were not dissolved (except for what goes into solution by a few seconds of shaking). The other substances were in solution. The value of −ΔpH is seen to be the difference between the pH of the mixture and that of the ampholyte.

	gelatin 0.25 g.	casein 0.15 g.	albumen 0.25 g.	peptone 0.25 g.	alanine 0.15 g.
pH of dye solution	4.73	4.73	6.42	5.35	5.96
pH of ampholyte in water	4.73	4.73	6.42	5.30	5.92
pH of ampholyte in dye	4.38	4.30	5.87	5.03	5.76
−ΔpH	0.35	0.43	0.55	0.27	0.16

results are given. There are also included a few experiments on other substances, such as a sample of egg albumen, a soluble preparation probably containing albuminate, a sample of Leitz's peptone and one of alanine. These last three substances were all soluble. The insoluble materials used were casein and gelatin. In these experiments

the pH which a weighed amount of the ampholyte gave to 25 cc. water was measured. Then 25 cc. of a crystal violet solution, 1.5 grams per liter, were adjusted to this pH. A weighed amount of ampholyte was then added and the pH of the resulting mixture determined after equilibrium was established.

The above results indicate a mechanism analogous to that of certain well known ionic reactions. In the system acetic acid and lead salt (e.g. $PbCl_2$) we have the following equilibria:

$$HAc \rightleftharpoons H^+ + Ac^-$$
$$\rightarrow \quad +$$
$$\tfrac{1}{2}\ Pb^{++}$$
$$\downarrow$$
$$\tfrac{1}{2}\ Pb\ (Ac)_2$$

The un-ionized $Pb(Ac)_2$ removes acetate-ion with increase in hydrogen-ion concentration. This effect is of a different order of magnitude

TABLE 6

Showing the ordinary "salt effect" in the change of pH on acetate buffer solutions,* and comparing the magnitude of this with that of the effect of the specific Pb ion which forms an un-ionized salt with acetate ion.

pH of original buffer	Barium Salt		Potassium Salt		Lead Salt	
	pH	—ΔpH	pH	—ΔpH	pH	—ΔpH
2.18	2.19	—0.01	2.18	——	1.99	0.19
2.68	2.65	0.03	2.67	0.01	2.49	0.19
3.26	3.21	0.05	3.23	0.03	2.95	0.31
3.76	3.68	0.08	3.74	0.02	3.52	0.24
4.28	4.21	0.07	4.25	0.03	4.09	0.19
4.77	4.71	0.06	4.74	0.03	4.60	0.17
5.25	5.19	0.06	5.20	0.05	5.12	0.13

*The first two solutions were pure acetic acid, the other five mixtures of acetic acid and sodium acetate with a total acetate concentration of about 0.05 normal.

from that of the well known "salt effect" shown by such salts as $BaCl_2$ or KCl, tho the mechanism is thought to be of the same nature. Table 6 gives data on acetate buffer mixtures treated with $PbCl_2$, and, for comparison in order of magnitude, includes similar data on the same acetate mixtures treated with $BaCl_2$ and KCl. In all cases, to three 25 cc. portions of the acetate mixtures 0.1 g. $BaCl_2.\ 2H_2O$, 0.06 g. KCl and 0.05 g. $PbCl_2$ were added respectively. These respective amounts of the first two salts are about chemically equivalent and are equal to nearly 2.5 times the amount of the lead salt added in equivalents, tho the effect of the last salt is seen to be enormously greater than that of the other two.

Discussion

The only alternate explanation of the behavior of the systems reported in this paper which occurs to the authors is the following. Basic dyes may hydrolyze in water according to the equation

$$DCl + HOH \rightleftharpoons DOH + HCl$$

If the proteins or nucleic acid should tend to adsorb the neutral dye base, this hydrolysis would proceed with increase in HCl and consequent lowering of pH. There are several reasons why this picture is inadequate. In the first place the effect is just as great when the substances which would have to act as adsorbents are in solution as when they are not. In the second place the actual increase in hydrogen-ion concentration is much greater at lower pH-values than at higher ones, in regions where the hydrolysis of the dye can hardly be considered to take place at all and where there would be no free dye base. Another objection will be suggested but will not be discussed as it involves added experimental work which is not here reported. It is the fact that the removal of neutral dye base and the increase of hydrogen-ion should result in an increase in conductivity of the solution. Actually it has been found that the conductivity DECREASES.[1] This latter effect is entirely in agreement with the chemical mechanism postulated in the introduction.

The present authors have become more and more convinced that staining reactions involve primarily chemical forces. They have no quarrel with the term adsorption except its indefiniteness, and their attempts to get at a concrete mechanism of staining may be interpreted as attempts to uncover the mechanism of adsorption. In colloid chemistry, tho this term is still very generally used, it is more and more taking on a chemical significance and the cases of adsorption which have been studied in most detail have yielded results which have pointed more and more definitely to the action of chemical forces. It has long been known that proteins in their acid and base binding behavior show a definite maximum binding capacity independent of further change of pH, i.e. the Freundlich isotherm, if it holds at all, holds only within restricted pH limits. These limits are sometimes wide and consequently there are still some who do not consider these reactions as essentially neutralizations. The ordinary monobasic acids bind bases significantly only thru a pH-range of four units. Phosphoric acid, however, binds base thru 11 pH-units and yet we do not speak of this binding as adsorption. It is true that in this case there are distinct breaks in the curve, since one hydrogen is almost completely neutralized before the next begins to ionize, but

[1] Stearn, A. E. Unpublished Data.

it is very easy to prepare a mixture of simple acids which will bind base uniformly from a pH of 2 to one of 13 where there would be no question but that the mechanism was one of neutralization. A polybasic acid with ionization constants rather close together would show this same behavior, but would eventually be completely neutralized and show a final maximum binding independent of further pH change. Stearn[2] has shown that the equivalent weight of gelatin is the same whether obtained from the maximum binding of acids and bases determined by electrometric titration, or obtained by conductivity titrations, regardless of whether the gelatin be titrated with acid or base or the acid or base titrated with gelatin solution. This is of interest since in the one case one ends the titration at very high or very low pH values, while in the other case one ends the titration near the isoelectric pH of the gelatin.

With inorganic colloids Pauli and Schmidt[3] have shown that the acid binding of alumina reaches a maximum independent of pH. Rabinovich and Dorfmann[4] state that adsorption by As_2S_3 does not follow the Freundlich isotherm but reaches a definite maximum. Rabinovich and Kargin[5] show that adsorption and chloride ion displacement by Fe_2O_3 sols does not follow the ordinary adsorption laws but acts much more as a chemical "displacement reaction" analogous to the reactions studied in this paper.

Frumkin[6] has shown that thoroly activated charcoal will adsorb neither acid nor alkali from solution. When saturated with oxygen it adsorbs acids and when saturated with hydrogen it adsorbs alkalis. These findings are explained on the basis of an electro-chemical theory of adsorption. Kolthoff and Tohru[7] have reported a very similar behavior on the part of platinized platinum.

Even such an apparently purely physical phenomenon as the adsorption of crystal violet on glass[8] has been shown to reach a defi-

[2]Stearn, A. E. The determination of the equivalent weight of proteins. J. Gen. Physiol., 11, 377. 1928.

[3]Pauli, W. and Schmidt, E. Physical chemical studies of aluminum oxide solution. Zts. Physik. Chem., 129, 199. 1927.

[4]Rabinovich, A. I. and Dorfmann, V. A. The coagulation of colloids by electrolytes. II. Conductivity studies of the process of coagulation of arsenic sulfide sols. Zts. Physik. Chem., 131, 313. 1928.

[5]Rabinovich, A. I. and Kargin, V. A. The coagulation of colloids by electrolytes. III. Potentiometric titration of the coagulation of ferric oxide sols. Zts. Physik. Chem. 133, 203. 1928.

[6]Frumkin, A. The influence of oxygen and hydrogen on the adsorption of electrolytes by charcoal. Paper read at the National Meeting of the American Chemical Society, Columbus, Ohio, April 30–May 2, 1929.

[7]Kolthoff, I. M. and Tohru, K. The measurement of the hydrogen-ion concentration in unbuffered solutions. I. The adsorbent properties of platinized platinum. Paper read at the National Meeting of the American Chemical Society, Columbus, Ohio, April 30–May 2, 1929.

[8]van den Grinten, K. Adsorption and cataphoresis. J. Chimie Physique, 23, 209. 1926.

nite limit when a monomolecular layer has been bound. The molecules in this layer seem to be definitely oriented, indicating dominance of specific chemical forces.

Chemical theories have been criticized as postulating definite chemical compound formation, by which is probably meant systems which give identical total analyses. Regardless of whether one consider this postulate a necessary consequence of chemical theory, one need not expect the same definite chemical compound under a variety of conditions. If there are a sufficient number of possible compounds sufficiently similar, one may get a total impression of non-definiteness in purely chemical reactions. As ordinarily interpreted in such connections, this criticism seems to assume that all chemical reactions tend to go to completion, which is seldom the case.

An example should make this clear. Such a simple system as oxalic acid and alkali hydroxide will yield at least three definite compounds depending on the extent to which the acid is neutralized; namely the tetroxalate, the binoxalate and the normal oxalate. If one neutralizes with an indefinite amount of alkali and crystalizes the product, one may get various mixtures of these salts, which, *apparently*, may vary almost continuously in composition, tho few would question the fact that such a neutralization is typically chemical. More complicated systems may seem more indefinite when viewed superficially. Definiteness of chemical reaction should not be confused with stability. Even such a weak acid as acetic will completely liberate hydrocyanic acid from cyanides, but in its turn it will be completely liberated from acetates by HCl.

STAINING OF NEGRI BODIES

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ABSTRACT.—**The following procedure for staining Negri bodies in sections is based on methods previously described by MacNeal, by Haynes, and by Richter:**

Fixation:

1. **Zenker's solution 4 hours at 37°C or Dominici's 3 hours.**
2. **70% alcohol, 12 to 18 hours at room temperature.**
3. **80% alcohol, about 5 to 6 hours.**
4. **90% alcohol, about 4 to 6 hours.**
5. **Absolute alcohol about 16 hours.**
6. **Ether and absolute alcohol *aa*, about 8 hours.**
7. **16 to 24 hours in the following mixture: celloidin 1 g., methyl salycilate 25 cc., abs. alcohol 25 cc., ether 25 cc.**
8. **Chloroform and paraffin, 2 to 3 hours.**
9. **Paraffin, 2 hours.**
10. **Paraffin, 1 to 1½ hours.**
11. **Embed.**

Staining:

1. **Cut sections 4 to 5 μ.**
2. **Bring section to water and cover with Lugol's iodine for 10 minutes.**
3. **Decolorize with a 2% sodium thiosulfate (hypo).**
4. **Wash thoroly with water.**
5. **Cover with a mixture of equal parts of 0.5% phloxine and 1% eosin Y (National Aniline brand) and leave for 15 minutes.**
6. **Wash with water and stain 2 to 5 minutes in 0.1% azure B (National Aniline).**
7. **Wash with 96% alcohol and decolorize in a mixture of 2 parts absolute alcohol with 1 part clove oil, ordinarily for not more than ½ to 1 minute.**
8. **Dehydrate rapidly, clear, and mount in Yucatan Elemi.**

The staining of Negri bodies is not usually regarded as a difficult operation.

When, however, one goes a little deeper into the matter than can be attempted in the average text book, one is somewhat surprised to note that not only does a variant of the favorite methods appear almost every six months, but that the various authors who have made a special study of the matter, tho they often consider that there

is not much difficulty in revealing these bodies, have to admit that they cannot get good results with methods other than their own favorites. Thus Williams[1] in America finds that whilst Giemsa is good for sections and fuchsin-methylene-blue for smears, Mann's method is a failure. In France, Mann's method is the most popular, and in India and in Germany other methods are described. This state of affairs hardly supports the contention that Negri bodies are easily stained.

Some years ago, the senior author undertook a somewhat more prolonged research than the subject perhaps warranted. Numerous dye combinations were tried out, as well as various fixatives. The methods tried were very numerous and included most of those used at the present time, and in addition numerous stains not normally in use, notably the oxyazime and azo-carmin series. As however, all were found to be somewhat capricious in our hands, it will perhaps suffice to say that other than the method about to be described, a considerable degree of success was obtained with the Campanacci, Goodpasture and Epstein[2] methods. The results with the last named are often very beautiful but it cannot be used as a routine method, since it requires great care and concentration for its successful application.

Finally a satisfactory method was evolved which is not in the least original, since it is based on the work of MacNeal, of Rachel Haynes, and of Richter.[3] Indeed it is only of interest in view of the bearing it has on the standardization of stains, and inasmuch as it emphasizes the necessity for the accurate description of stains in all accounts of staining methods. During the investigation various facts were noted not directly bearing on the actual staining reaction. For example, it would seem that the successful fixation of Negri bodies must be a

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[1]Cited from Park, W. H. and Williams, A. W. Pathogenic Micro-organisms, *7th ed.*, Lea and Febiger, Phila.; see p. 615 ff. See also Williams, A. W. and Lowden, M. W. The etiology and diagnosis of hydrophobia. J. Inf. Dis., **3**, 452–483. 1906.

[2]Campanacci, D. Un nuovo metodo pratico di colorazione dei corpi di Negri. Sperimentale, **79**, 665. 1925. Cited from Trop. Dis. Bul., **23**, 199. 1926.

Goodpasture, E. W. A study of rabies, with reference to a neutral transmission of the virus in rabbits, and the structure and significance of Negri bodies. Am. J. Path., **1**, 547. 1925.

Epstein, H. Beitrag zur zytologischen Diagnose der Lyssa. Centbl. f. Bakt., I Abt. Orig., **92**, 71. 1924.

[3]MacNeal, W. J. Methylene azure B. Staining sections of hematopoietic tissues. Am. J. Path., **2**, 578. 1926.

Haynes, Rachel. Azure stains. Stain Techn., **1**, 107. 1926.

Richter, W. N. A modified methylene azure B stain for sections of human hematopoietic organs. Arch. Path. & Lab. Med., **4**, **5**, 773. 1927. Cited from J. Lab. & Clin. Med., **14**, 82. 1928.

quick process, and any prolongation beyond eight hours gave bad results. Picric acid fixatives with the exception of B15, the picric formal urea formula mentioned by McClung,[4] proved useless and the best fixatives of those tried were Zenker's and Dominicci's.[5]

A paraffin-celloidin embedding gave the best results and is used in all cases (it is regretted that the name of the author of this method has been forgotten). Mounting in paraffin alone saves time but is liable to result in poor specimens. Mordanting with copper acetate sometimes greatly improves the picture and this was especially true before fuchsin staining. Indeed the fact that Negri bodies stain moderately well by Alzheimer's[6] method for degeneration bodies may not be without significance.

Other mordants such as ammonium molybdate gave at times a fine result, especially when used in combination with acid fuchsin and hematoxylin, but the optimum method for this combination could not be discovered and its use was abandoned as sufficient time had been wasted on its trial.

Successful specimens showed more Negri bodies especially of the small type than any other method. The methods used were modifications of Held's[7] molybdic hematoxylin, acid fuchsin, aurantia, or picric acid.

Finally however a phloxine-eosin followed by one of the azures was standardized.

Several phloxines had to be tried but eventually that of the National Aniline Company was used exclusively. That of Grübler was too variable and indeed at times did not seem to be phloxine at all as far as its reaction to the method was concerned. One specimen labelled "magdala red" (actually a phloxine or erythrosine) was tried with good results, but the more expensive and presumably true magdala red was useless. The National Aniline phloxine had, however, to be used in dilute solutions, and indeed was regarded as useless until we obtained this information from the Stain Commission.[8]

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[4]McClung, C. E. Handbook of Microscopical Technique. Hoeber, New York, 1929. See p. 424–5.

[5]Dominicci's fixative was obtained from: Manouelian, Y. and Viala, J. "Encephalitozoon rabiei" parasite de la rage. Ann. Inst. Past., 38, 258. 1924. (The non-alcoholic formula is the one used.)

[6]Alzheimer's stain for fatty degeneration granules; quoted in: Mallory and Wright's Pathological Technic, *8th ed.* (Saunders, Phila., 1924), p. 160.

[7]See Romeis' Taschenbuch der Mikroskopischen Technik, p. 442.

[8]See Conn, H. J. and Holmes, W. C. Fluorescein dyes as bacterial stains; with special reference to their use for soil preparations. Stain Techn., 1, 87. 1926. Certain factors influencing the staining properties of fluorescein derivatives. Id. 3, 94. 1928.

The admixture of eosin Y seemed to render the darker red (phloxine?) of the Negri body more conspicuous against the light red cytoplasm. Various azures can be used. We prefer azure B, but this seems a matter of taste, and a trace of methylene blue in the mixture improves the picture to some eyes. Azure C did not suit the method at all.

The addition of formalin to the phloxine-eosin stain (as first suggested by Ellerman[9] and Paul and Schweinburg[10], just before use gives a more highly differential staining with the blue dye, but makes the actual process of differentiation harder.

Iodine in this method, as well as removing the sublimate of the fixative has a mordanting effect, and should the first sections stain badly, leaving the others for an hour or even more in dilute Lugol's iodine often gives an excellent result. (Compare use by Masson[11] in his modification of Mallory's phosphotungstic hematoxylin method).

Colophonium must be added to the clove oil and alcohol if formalin is added to the phloxine-eosin stain. It slows the action of the decolorizer but improves the picture.

Method—Fixation

Fix reasonably thin portions of hippocampus in Zenker's or Dominici's fluid for four or six hours. (*Note:—If the Hippocampus is to be pared down, one should remember that the ends, especially the anterior, contains the majority of the bodies.*)

1. Zenker's 4 hours at 37°C or Dominici's 3 hours at 37° C.
2. 70% alcohol overnight at room temperature, usually about 12 to 18 hours.
3. 80% alcohol, about 5 to 6 hours.
4. 90% alcohol, about 4 to 6 hours.
5. Absolute alcohol overnight, usually about 16 hours.
6. Ether and absolute alcohol *aa* about 8 hours.
7. Celloidin mixture 16 to 24 hours.[12]
8. Chloroform-paraffin, 2 to 3 hours.

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[9]See Romeis' Taschenbuch der Mikroskopischen Technik, p. 231.

[10]See Krause's Enzyklopädie der Mikroskopischen Technik, (Urban & Schwarzenberg, Berlin, 1927) p. 2175.

[11]See Landeron's Precis de Microscopie (Masson et Cie.) *4th ed.*, p. 456.

[12]

Celluloid II	1 g.	
Methyl salycilate	25 cc.	Celloidin
Abs. alcohol	25 cc.	mixture
Ether	25 cc.	

9. Paraffin 1, 2 hours.
10. Paraffin 2, 1 to 1½ hours.
11. Embed.

Staining—Phloxine-Eosin-Azure B

1. Cut sections 4 to 5 μ.
2. Bring section to water and cover with Lugol's iodine for 10 minutes.
3. Decolorize the iodine with a 2% sodium thiosulphate (hypo).
4. Wash thoroly with water.
5. Cover with a mixture of equal parts of 0.5% phloxine and 1% eosin Y (National Aniline brands) and leave for 15 minutes.
6. Wash off with water and stain for 2 to 5 minutes in 0.1%, methylene azure B (National Aniline brand).
7. Wash off with 96% alcohol and decolorize in a mixture of 2 parts absolute alcohol and 1 part clove oil. This solution acts very quickly and care must be taken not to prolong this stage; ½ to 1 minute is usually sufficient.
8. The section is now rapidly dehydrated and cleared and mounted in Yucatan elemi.

As an alternative method of staining, Goodpasture's method is used, but is preceded by mordanting in copper acetate for one hour. Zenker's fixative is the best for this method. More rapid fixation can be obtained as follows:

3% Sulpho-salicylic acid and 5% acetic acid in absolute alcohol (or 7%).

Fix for 18 hours (i. e. overnight) and then transfer to absolute alcohol for about 4 to 6 hours,

Then celloidin mixture, paraffin baths and embed.

By this method the picture is not so good, however.

Conclusions

A method has been described which in the author's opinion stains Negri bodies with more certainty than the majority of other methods. It does not perhaps show the various details of the so-called structure as well as do other methods. In this method the addition of formalin to the phloxine-eosin makes for more intense nuclear staining and renders the "Innenkorper" most distinctive. Phloxine would seem to be the substance in impure eosins which has the greatest affinity for the Negri body, tho it is not certain which of the two phloxines was employed, nor whether better results could be obtained with the

other type. Rose bengal, rhodamine (acid forms) and various brands of eosin from numerous manufacturers were tested without the same success. From an empirical standpoint, one is led to believe that Negri bodies have certain affinities with nuclear substance, since the great difficulty in staining them satisfactorily lies in differentiating the nuclear stain, and in the fact that in some stain combinations they are commonly stained in the same tint as the nucleolus.

Staining methods for cell inclusions, and certain methods used for degeneration products are also of value.

Yucatan elemi balsam (Southgate)[13] is one of the best mountants for eosin methylene blue specimens, tho curiously enough Canada balsam made with xylol saturated with salicylic acid is also very good. (This is rather contrary to the accepted opinion that fading is due to acid balsam.) Finally since this method is only a modification of an old one, we may assume that it will not meet with favor in the eyes of those who prefer a method based on different staining reagents, and that the ideal method is yet to be found.

[13]Southgate. A suggested substitute for Canada balsam as a mounting medium. Brit. J. Exp. Path., 4, 44. 1923.

A MODIFICATION OF MAYER'S HEMALUM

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A survey of histological literature shows that Delafield's hematoxylin is one of the most widely used histological stains. Personal interviews with workers in this field elicit comments which seldom appear in print. Most workers confess that it usually requires several unsuccessful attempts to make a usable stain. The experienced technician is no more successful than the self-taught beginner. I have examined, at various schools, preparations stained with so-called Delafield's stain and the range of colors and degrees of muddiness found were surprising. A somewhat greater degree of certainty and uniformity is desirable in a stain which is to be used by classes in elementary histology. Mayer's hemalum has not enjoyed the popularity which it deserves perhaps because the formula given in a widely used text is incorrect. This was found to be a source of discouragement to self-taught students in some smaller colleges.

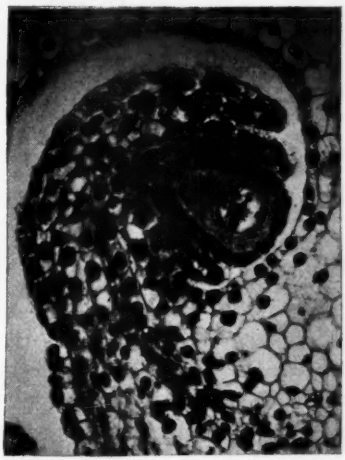

FIG. 1. *Lilium tigrinum*. Ovule, with megasporocyte shortly before heterotypic division. Bouin fixation, stained 15 minutes in modified hemalum. × 180.

I have been using a stain based on the Mayer formula but prepared by a method which brings about optimum "ripening," affording maximum intensity and selectivity in the freshly-made stain. The procedure is as follows: Dissolve 50 grams of alum $Al_2(NH_4)_2.4SO_4$ in a liter of boiling water. Remove from the hot-plate and add 1 g.of hematoxylin, (obtained from Coleman & Bell, National Aniline Co. or MacAndrews and Forbes).[1] Add 1 g. $NaIO_3$, cool and filter. The stain should be filtered when ever a "metallic" scum is present. The solution is best when fresh, but its staining properties are retained for at least six months. The keeping qualities are now being tested.

[1]Hematoxylin with the certification number FH7 was employed in this work. It will be recalled that Mayer specified hematein in this staining fluid. Inasmuch as a satisfactory hematein is not now available the use of hematoxylin with $NaIO_3$ is to be preferred. (*See, however, page 97 of this issue.—Ed.*)

The slide to be stained is transferred from water to the staining fluid. It is washed in distilled water, then in tap water (or 1/100 Na_2CO_3), and again in distilled water. Finally it is dehydrated and cleared as usual. An aqueous or alcoholic counterstain may be used. This is primarily an histological stain. The phloem and pith of corn stem may be stained in 15 minutes. The tissues of Bryophytes take a good stain. A differential staining of mycellium and host may be accomplished in some materials, i.e. an endotrophic mycorrhiza on orchid, *Ustilago zeae*, *Puccinia coronata* and many others. The

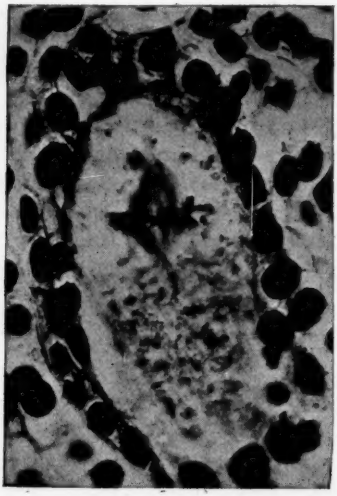

FIG. 2. *Lilium tigrinum*. Embryo sac, heterotypic division. Note disjunction of chromosomes. Bouin fixation, stained one hour in acid hemalum. × 500.

applicability for this purpose, however, is by no means general. Lily ovary stains in 15 to 30 minutes and the nuclear selectivity is entirely adequate for preparations to be used in elementary botany (see Fig. 1).

Using the above stain as a basis, we can prepare various "acid hemalums" in which the nuclear selectivity is increased. The alum can be increased to saturation and 2 to 5% glacial acetic acid added. The results vary with the fixation used on the tissues. A concrete case is illustrated by Figs. 2 and 3. The lily ovary was killed in Bouin's solution. The stain contained alum to saturation and 3% acetic acid, and the staining period was one

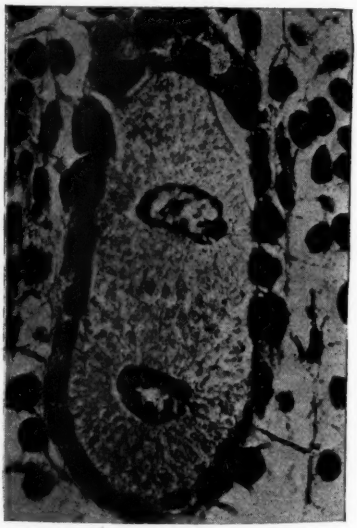

FIG. 3. *Lilium tigrinum*. Embryo sac with two daughter nuclei after heterotypic division. Bouin fixation, stained one hour in acid hemalum. × 500.

hour, without mordanting or differentiation. The staining of the somatic nuclei, the chromosomes and the spindle area, is satisfactory for general botany, tho by no means good enough for cytological work. Considering the number of slides used (and broken) in freshman botany, and the more or less inexperienced help available for slide-making, the convenience and certainty of these stains makes them valuable.

A SHORT METHOD OF CLEARING PLANT TISSUES FOR ANATOMICAL STUDIES

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In connection with an investigation of the vascular anatomy of certain flowers, the writer has developed a time-saving method which has a second advantage in showing all parts in their proper relation to one another. It is an extension of a method described by J. T. Buchholz and A. F. Blakeslee[1] for the study of whole pollen-tube populations within stigmas of Datura. Their method was one of dissection, the material being first scalded and fixed, then dissected and stained. Lactic acid was used as the clearing and mounting medium. The writer uses lactic acid directly upon fresh material, killing, clearing and mounting it without disturbing the specimen. This procedure is simple, inexpensive and applicable to most plant tissues. It is therefore recommended for classroom work.

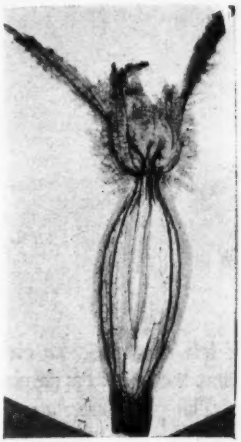


FIG. 1. A young pistillate cucumber flower. A thin slice has been taken from upper and lower surfaces so that the specimen lies flat under the cover glass.

The material to be studied is placed in lactic acid of a concentration of about 75%. Whole blossoms or large parts such as the pistil may be immersed in the medium in an open watch glass. Sections of fresh material, cut fairly thick by hand, may be mounted in the acid on a slide and the cover glass applied. In either case, the specimen is then placed in a constant temperature oven and kept at about 54° C until clear. The time required varies with the material. Sections of floral parts, young fruits or succulent stems become clear in two or three hours. Whole parts or thick wedges of such material have required, in the writer's experience, about twelve hours. It is often convenient to leave them over night.

If permanent slides are desired, the lactic acid may be thickened by slow evaporation in the oven until it is almost hard. The edges of the preparation may then be sealed. For this purpose, a mixture of gum

[1] J. T. Buchholz and Blakeslee, A. F. Pollen-tube behavior with reference to sterility in Datura. Mem. Hort. Soc. N. Y., 3, 245–260. July, 1927.

mastic and paraffin, applied with a hot iron wire,[2] has been found suitable. As lactic acid is hygroscopic, the slides become very sticky

FIG. 2. A thick longitudinal section thru the base of a young cucumber fruit, showing the arrangement of vascular strands.

if left unsealed. In cleaning up such slides it is useful to remember that lactic acid is soluble in alcohol.

The photomicrographs shown were taken from slides prepared in this way, without staining or any further treatment.

[2]This method of sealing is not new and was not originated by the writer.

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked. Thru coöperation with the Wistar Institute of Anatomy and Biology of Philadelphia, these same abstracts are to appear on the Laboratory Service Cards of that institute. Those desiring some of these cards without subscribing for the entire Bibliographic Service of the Wistar Institute, can order them direct from STAIN TECHNOLOGY, at a price of 6 cents each.

LENTZE, HRCH. Anisol statt Zedernöl als Immersionsmittel nach Becher. Zts. Wis. Mikr., 45, 200–202. 1928

Anisol is proposed instead of cedar oil for immersion oil. The former is a phenyl-methyl-ether, ($C_6H_5 \cdot O \cdot CH_3$). The zoologist Becher considered anisol as good as cedar oil without having the unpleasant quality of stickiness and undesirable thickness after short exposure to air. According to Becher the refractive index of anisol is 1.5103 and that of thick cedar oil usually indicated as 1.51 or 1.52, and that of thin cedar oil as 1.504. It is a thin clear liquid, which may darken, but does not influence its refractive qualities. Altho anisol evaporates, it is recommended to wipe it off with a cloth or filter paper, which absorbs it. It does not damage lenses, metal plates, etc.

Blood smears stained according to the May-Grünwald-Giemsa method have not faded after soaking in it for a day. The thinness of anisol is a disadvantage because it floats down when used on a slanting stage. It becomes quite stable, however, after coming in contact with the lens. The author recommends it highly for general use after a two-years' experience. Anisol can not be used for demonstrations on account of its volatility.

FARCONI, ALDO. Il metodo Petragnani per le ciglia batteriche e le sue applicazioni alla colorazione de tessuti animali. Bull. d'Histol. Appl. 6, 306–314. 1929.

The writer describes the method for staining bacterial flagella proposed by Petragnani in 1922. The first step is mordanting of the carefully prepared bacterial films in a mixture of the following solutions:

Solution A		Solution B	
Tannic acid (purest)	7 g.	Potas. Alum (crystals)	3 g.
Ferric chloride	35 g.	Zinc acetate (crystals)	0.5 g.
Ethyl alcohol	35 cc.	Acetic acid, glacial	3 drops
Distilled water	15 cc.	Distilled water	10 cc.

After washing the preparation is stained cold in Ziehl's carbol fuchsin, gentian violet, or in a saturated alcoholic solution of crystal violet in anilin water. (Formulae not given.) He shows that the same technic may be used with fine results on fixed paraffin sections of various animal tissues. Photomicrographs and colored drawings are given to illustrate the effects obtained.

BRILMYER, G. J. A nuclear and differential stain combined. Science, 68, 114. 1928.

1. Stain sections in Delafield's hematoxylin for 5 minutes.
2. Pass thru distilled water to remove excess stain.
3. Stain in 0.2% aqueous solution of acid fuchsin for 1 minute.
4. Pass thru distilled water to remove excess stain.
5. Stain in the following solution for 2 to 3 hours:

Anilin blue (water soluble)	0.5 g.
Orange G	2.0 g.
Phosphomolybdic acid (1% aqueous solution)	100.0 cc.

6. Pass thru distilled water to remove excess stain.
7. Pass successively (rapidly) thru the following grades of alcohol: 36%, 70% and 95%.
8. Complete dehydration in absolute alcohol in one-half to one minute. (Water-free acetone may be used in place of absolute alcohol, with but little or no shrinkage of the cells.)
9. Clear in xylol.
10. Mount with cover glass.

With this staining nuclei will appear a rich red, epithelial cells pink, connective tissue blue and muscle red. Red blood cells will stain yellowish in veins and reddish in arteries. Colloid and mucus stain blue.

The staining seems to follow any fixation well. Sections stained by this method have not faded in five years.

The writer states there is nothing new in the procedure other than the method of combining Delafield's hematoxylin with Mallory's connective tissue stain. (The source or exact nature of the dyes employed is not given.)

CRAIGIE, JAMES. The demonstration of bacterial flagella. J. Roy. Micro. Soc., Ser. 3, 49, 9–13. 1929.

A more detailed report on essentially the same technic already reported from a different source (*Stain Techn.* 4, 31). After fixing the organisms with formalin, drying at 90°C and mordanting with Zettnow's mordant, there are three ways the flagella may be demonstrated: 1) by dark field examination; 2) by an ordinary bacterial stain following Gram's iodine solution; 3) by Zettnow's silver impregnation method followed by gold toning, as previously described, or by replacement of the silver with uranium. This last mentioned procedure, which was not mentioned in the earlier paper calls for immersion for about 15 minutes in 0.1% uranium chloride (or weaker), and after washing 2 to 5 minutes application of the following intensifier:

Pyrogallic or hydroxy-naphthionic acid	0.05 to 0.1	g.
Water	10	cc.
Liq. ammon. fort	5	drops

Rinse and stain with methyl violet for 5 minutes.

FOLY, J. O. Studies in stain technique. I. Flemming's safranin, gentian violet, orange G method, with a suggested modification. Anat. Rec., 43, 171–185. 1929.

1. Mordant the sections in 0.5% aqueous solution osmic acid for 30 minutes to one hour. This is omitted for material which has been fixed in fluids which contain osmic acid.
2. Thoroly wash in water and stain 1 to 3 hours or more in 1% aqueous safranin.
3. Rinse in water and differentiate the safranin in a 0.025 N solution of HCl acid. Allow the acidulated water to remain on the slide until the outline of the nucleus is definite and chromatin granules discerned. Do not remove too much stain at this point. Rinse in water.
4. Stain in a 0.27 to 0.3% solution of crystal violet in 7% alcohol for about 20 min.
5. Rinse in water and differentiate in 0.025 N solution of HCl acid. Differentiation is far more important than the time of staining, it should be stopped when the nuclei take a definite outline.
6. Rinse in water and put in Gram's solution for 1 to 3 minutes, or until the slides have turned a deep black.
7. Thoroly wash off excess iodine and immerse in a 1% solution of bichloride of mercury for 1 to 3 minutes, or until the sections have turned a bright blue.
8. Wash in water and blot excess, not letting the sections completely dry.
9. Immerse in 95% alcohol for 4 to 6 seconds and transfer to carbol-xylol (25 parts of phenol to 75 parts of xylol) while the violet is still coming out in clouds.
10. Leave in carbol-xylol from 15 seconds to several minutes or until proper differentiation, that is when heads of spermatozoa, metaphase, anaphase and telophase chromosomes and basophilic nucleoli are definitely red, and the spireme of prophase is a deep blue.
11. Wash in xylol and stain in a 1% solution of orange G in clove oil few seconds to one minute.
12. Wash with pure clove oil and xylol. Mount in balsam.

The author used following fixatives: Flemming's, Bouin's Gilson's, and Carnoy-Lebrun's. Mordanting with potassium permanganate was also tried; it cuts down the staining time for safranin and intensifies it in the chromatic structure, but fixes the violet to such a degree that it is almost impossible to differentiate it further. Unless one wants to intensify the violet in the achromatic structure one should not use permanganate. The dyes employed are from several sources; exact nature and dye content are carefully specified.

This triple stain gives the usual Flemming's differentiation, with clearer results perhaps.

LEPIK, E. Differential staining of the Peronosporaceae. Phytopath. 18, 869–872. 1928.

Paraffin is dissolved out of sections with xylol or turpentine; and they are passed thru 100% and 96% alcohols. They are then treated 10 to 15 minutes in the following:

Solution No. 1:

Phenol	10 g.
Conc. lactic acid	10 cc.
Conc. glycerin	20 cc.
Alcohol (96%)	20 cc.

They are then stained two hours in Solution No. 2:

Cotton blue 4B (thought to be identical with oxamin blue 4BX or dianyl blue G)	0.02 g.
Safranin	0.1 g.
Solution No. 1	100 cc.

(These stains were obtained from the firm of Bender and Hohlbein of Zurich and Munich.)

Differentiate under the microscope, overstaining being controlled by Solution No. 1. Wash in absolute alcohol. Treat 20 to 30 minutes in a weak solution of safranin in clove oil. Differentiate under microscope with pure clove oil. Clear in xylol and mount in balsam.

This method has been used with *Plasmopara viticola* in the leaf tissue of *Vitis vinifera*. The mycelium shows blue and the tissue red.

STEGALL, P. A. A method of differential staining of the human brain. Anat. Rec. 42, 399. 1929.

The method presented is intended for staining sections of the whole human brain. *Solution 1:* Add enough ammonia to one-half pound of methylene blue to make the mixture the consistency of soft putty. To this add three quarts of cedar oil and boil the mixture for ten minutes stirring vigorously. Cool for four hours and strain thru a cloth. *Solution 2:* Dissolve 40 g. of mercurochrome in one quart of water containing 100 g. of soap. Determine by experiment the proper time for staining in solution 1 by immersing four slides in the solution and removing one every 5 minutes until the white matter is stained the desired depth. The oil in this bath removes the paraffin from the section. Allow the slide to drain. Place a soft piece of cotton cloth over the section and blot gently. Immerse the slide in strong soap solution for thirty seconds, wash in water, and stain in the red dye until the gray matter is the desired color. Allow the section to dry (not longer than ten minutes). Put a teaspoonful of cedar oil at the end of the slide and spread it over the section with a piece of fenestra, using the fenestra for a cover. Trim off the edges with a razor blade, smooth the surface with a squeegee roller, wipe off the excess oil and put tape around the edges of the slide.

UGRIUMOW, B. (LENINGRAD.) Praktische Winke zur Azur II-Eosinfärbung an Schnittpräparaten. Zts. Wis. Mikr. 45, 191–192. 1928.

Azure II-eosin is recommended as critical blood stain in pathologic anatomy. The author finds that azure II-eosin according to Nocht Maximow works decidedly better than the May-Grünwald-Giemsa stain or the Panchrome stain.

Method: Fix in Zenker formol, embed in celloidin paraffin. (Intermediate stage chloroform, not oil.) Prepare azure II- 1:1000 (Hollborn) and eosin B A extra Höchst 1:1000. The pH of the solution should be about 6. 3 to 6.6. Mix 1.5 to 1.6 cc of the eosin solution with 1 cc of the azure solution and add 10 cc of water. Stain 10 to 12 hours changing few times. Differentiate with acetone. If the coloration is too basophilic use acidulated acetone (2 to 5 drops of glacial acetic acid to 20 drops of acetone.) From acetone transfer to xylol and Canada balsam. Do not use neutral Canada balsam because sections will fade in it after 2 to 3 weeks, while otherwise they keep about a year.

Neutral granules should be reddish violet, eosinophiles bright red, the cytoplasm of the plasma cells dark blue, lighter in immature ones, the promyelocytes grayish blue with a few carmin red granules, the nuclei dark blue and the nucleoli bluish gray or redish.

WOLF, JEAN. Sur la nature della coloration histologique et sur la methode de contact. Bull. d'Histol. Appl. 6, 259–267. 1929.

Celloidin sections, as for example, of the elastic cartilage of a bovine ear, are laid between thin sheets of celloidin stained in Böhmer's, Delafield's or Ehrlich's hematoxylin of 15-100μ thickness. Excess water is removed by filter paper and the whole is mounted in Canada balsam without dehydration. The slides are kept for about 24 hours at a temperature of 45°C, after which the balsam is dissolved by xylol. The slides are passed thru alcohol and water and differentiated in dilute HCl, or better in iron alum. After dehydration they are mounted in the usual manner.

The hematoxlyin should be ripe, but not overripe. All of the hematoxylin formulae give good results, except the ferric. Canada balsam should be neutral. The cellodin sheets placed between the sections may require 15 minutes to 24 hours staining, depending on their thickness, quality, and on the quality of the hematoxylin. The author uses formol fixation, but states that other methods can be used. It stains elastin, the organic substratum of the calcified substance, fibers, and muscle cells a deep black, while the background is unstained. Even the albuminoid material of the cartilage, usually hard to stain, is deeply colored.

INDEX TO VOLUME IV

Aceto-carmin 53, 54
  smears, method for making permanent 53
Acid fuchsin 12, 13, 21, 23, 30, 32, 34, 35, 62, 63, 81–86, 97, 98, 113, 115, 123, 133
Acriflavine 105
Acriviolet 105
Activity of the thyroid gland in relation to the staining reactions of the colloids. (abs.) 31
Alizarin 37
Alkanna roots, extract 38
Alkannin 40
Alum-hematoxylin 38–41
Amendment to constitution 71
Ammonia carmin 41
Ammoniacal hematein 46
Anderson, B. B.; J. Kisser and. Method of preparing thin cross and longitudinal sections of cotton fibers and its importance in cell wall research. (abs.) 93
Anilin 40
Anilin blue 82, 133
  picro- 95
Anisol statt Zedernöl als Immersionsmittel nach Becher. (abs.) 133
Apparatus for holding cover slips during fixation 59
Atmospheric oxidation, or dealkylation, of aqueous solutions of methylene blue .. 7
Aurantia 13, 96, 123
Azo acid yellow 74
Azure 123
Azure A 82, 83, 98
Azure B 82, 83, 121, 124
Azure C 124
Azure II 136
Azure-eosin 63
Azure II-eosin 136

Bacteria, detailed differentiation of, by means of mixture of acid and basic dyes at different pH-values 81
Bacteria, variation in staining character as related to reserve food material within the organism 105
Bacteria and mitochondria, double staining of, in plant tissues 13
Bacteria and yeasts, some staining methods for 21
Baer, Flora. An apparatus for holding cover slips during fixation 59
Baginski, S. Sur la detection histochimique de l'adrenaline. (abs.) 29
Basic fuchsin 3, 11, 12, 21–24, 31, 33, 98, 108, 133
Belling, J. A method for the study of chromosomes in pollen-mother-cells. (abs.) 94
Bismarck brown Y 98
Bowen, Robert H. A new staining dish for handling coverglass preparations. ... 57
Bratiano, S.; P. Galesesco and. Coloration des graisses par l'extrait alcoolique de *Daucus carota*. (abs.) 62
Brazilein 38
Brazilin 38, 96
  iron- 94
Brilliant cresyl blue 70
Brilliant croceine 74
Brilliant green 23, 24, 36, 113, 114
Brilmyer, G. J. A nuclear and differential stain combined. (abs.) 133

Carbol acid fuchsin 82
Carbol fuchsin 21–25, 32
Carbol gentian violet 82
Carbol methylene blue 24
Carmalum 93

Carmin 32, 38, 40, 41, 44
aceto- 53
lithium 62
Carotin 62
Carrots, (*Daucus carota*) extract 62, 94
Cartwright, K. St. G. A satisfactory method of staining fungal mycelium in wood sections. (abs.) 94
Chaze, J. Sur le mode de formation et la détection des alcaloïdes dans la plantule de tabac. (abs.) 61
Chemical analysis of thiazin eosinates 49
Chlorination method for macerating woody tissues 29
Chrysoidin 96
Cochineal 37
Coloration des graisses par l'extrait alcoolique de *Daucus carota*. (abs.) 62
Coloration des graisses par quelques pigments naturels. (abs.) 94
Coloration panotique d'exécution rapide permettant d'opérer sur la même préparation l'examin cytologique, l'examin bactériologique, et la recherche du bacille de Koch. (abs.) 31
Commission on Standardization of Biological Stains, present members of 5
Constitution of, amendment to 71
Congo red 3, 31
Conn, H. J. Note on Loeffler's methylene blue 27
Progress in the standardization of stains:
Definiteness in staining formulae 33
Hematein and acid fuchsin 97
Influence of impurities upon dyes 68
Safranin 65
Stain specifications 1
Stains recently certified 2, 36, 70, 98
The history of staining: Logwood dyes 37
Cotton blue 4B 135
Cover-glass preparations, new staining dish for handling 57
Cover slips, apparatus for holding during fixation 59
Craigie, J. A method of staining bacterial flagella. (abs.) 31
The demonstration of bacterial flagella. (abs.) 134
Crystal violet 11, 12, 32, 69, 70, 82, 83, 91, 98, 112–116, 119, 134, 135
Cyanole extra 74
Cytology of Saccharomyces cerviciae. (abs.) 31
Cytological work, differentiation of safranin in 27

Definiteness in staining formulae 33
Delafield's hematoxylin 42
Demonstration of bacterial flagella. (abs.) 134
Detailed differentiation of bacteria by means of mixtures of acid and basic dyes at different pH-values 81
Dianyl blue G 135
Differential staining of the Peronosporaceae. (abs.) 135
Differentiation of bacteria by means of mixtures of acid and basic dyes at different pH-values 81
Differentiation of safranin in cytological work 27
Dimethyl thionin 7
eosinate 52
Dimethyl thionolin 7
Dolfini, G. Su un nuovo metodo di colorazione dei grassi. (abs.) 94
Dorner's spore stain 108
Double staining of mitochondria and bacteria in plant tissues 13
Doubrow, S. Un. procédé rapide de coloration des bacilles de Koch dans les coupes histologiques (abs.) 94
Dufrenoy, J. Double staining of mitochondria and bacteria in plant tissues 13
Dyes, influence of impurities upon 68
Dyes, proteins, and nucleic acid, reaction between 111

Elastic tissue staining 11
Emploi des colorants mixtes en technique histologique. (abs.) 63

Emploi du mélange rhodamine-blue de méthylène dans la coloration des tissus splénique et lymphoide. (abs.) ... 63
Eosin .. 23, 32, 35, 49, 50, 51, 52, 62, 63, 64, 73, 74, 82, 95, 113, 115, 125, 126
Eosin B ... 3
Eosin BA extra ... 136
Eosin Y ... 3, 81, 98, 121, 124, 125
Eosinates, thiazin, chemical analysis of ... 49
Erb, N. M. A rapid stain for direct microscopic examination of milk. (abs.) ... 61
Erika B ... 74
Erythrin X ... 74
Erythrosin ... 73, 74, 98, 123

Färbung zur Darstellung reifer Samenzellen im Hodenschnitt-präparat. (abs.) ... 62
Farconi, Aldo. Il metodo Petragnani per le ciglia batteriche e le sue applicazioni alla colorazione de tessuti animali. (abs.) ... 133
Fast green FCF ... 74
Fixateur cytologique particulièrement adapté aux tissues de insectes. (abs.) ... 61
Flemming triple stain ... 66, 67, 135
Fluorescein ... 74
Foly, J. O. Studies in stain technic. I. Flemming's safranin, gentian violet, orange G method, with a suggested modification. (abs.) ... 135
French, R. W. Elastic tissue staining ... 11
Fry, H. J. A paraffin method for serially sectioning a minute object in a known plane. (abs.) ... 93
Fuchsin, acid ... 34, 35, 70, 97, 98, 113, 115, 123
basic ... 64, 70, 98, 108, 133
carbol- ... 77
Fuchsin-methylene-blue ... 122

Galesesco, P. and S. Bratiano. Coloration des graisses par l'extrait alcoolique de *Daucus carota*. (abs.) ... 62
Gaussen, C. Coloration panotique d'exécution rapide permettant d'opérer sur la même préparation l'examen cytologique, l'examen bactériologique, et la recherche du bacille de Koch. (abs.) ... 31
Gentian violet ... 22, 23, 24, 28, 32, 66, 70, 78, 81, 86, 91, 92, 105, 133
Giberton, A.; J. Millot, and. Sur utilization en histologie, des pièces conservées dans le formol, pour la mise en évidence des graisses. (abs.) ... 30
Giemsa stain ... 64, 122
Goldner, J. Résultats obtenus chez la seiche par l'emploi de divers colorants vitaux. (abs.) ... 62
Gram stain ... 78, 81, 86, 88, 91
note on ... 91
Green plant tissue, natural color preserved in sections ... 17
Guinea green B ... 74

Hadjioloff, A. Coloration des graisses par quelques pigments naturels. (abs.) ... 94
Une modification rapide de la méthode de Weigert-Pal. (abs.) ... 62
Harlow, W. M. A chlorination method for macerating woody tissues. (abs.) ... 29
Hausdorf, G. Färbung zur Dassellung reifer Samenzellen im Hodenschnittpräparat. (abs.) ... 62
Haynes, Rachel. Differentiation of safranin in cytological work ... 27
Heather, A. H.; J. H. Jordan and. Staining of Negri bodies ... 121
Hemacalcium ... 43
Hemalum ... 43
Mayer's modification of ... 127
Hematein ... 37, 43, 44, 45, 46, 97
ammoniacal ... 43
standardization of ... 97
Hematoxylin ... 12, 17, 18, 19, 30, 31, 32, 37, 38, 39, 40, 42, 43, 44, 45, 46, 62, 94, 96, 97, 123, 127, 128, 134, 136
alum ... 1, 38, 39, 40, 43, 61
Boetnner's ... 62
chrome ... 44
copper ... 44

crystalline 41
Delafield's 127, 133
glycerin 42
glycerinated alum 42
iron 12, 30, 44, 45, 94
iron alum 32
phosphomolybdic 45
phosphotungstic 46
staining with, history of 37
vanadium 45
Hewer, E. R. The activity of the thyroid gland in relation to the staining reactions of the colloids. (abs.) 31
History of staining: Logwood dyes 37
Holmes, W. C. Stain solubilities, Part IV 73
The chemical analysis of thiazin eosinates 49
The mechanism of staining. The case for the physical theories 75
Holmes, W. C. and E. F. Snyder. The atmospheric oxidation, or dealkylation, of aqueous solutions of methylene blue 7
Houcke, E. Emploi des colorants mixtes en technic histologique. (abs.) 63
Emploi des mélanges de fuchsines et de blues basiques pour la coloration histologique. (abs.) 63
Emploi du mélange rhodamine-blue de méthylène dans la coloration des tissues splénique et lymphoïde. (abs.) 63

Improvement in technic and results made in examining microscopically by razor section method 2000 malignant tissues. (abs.) 94
Indigo 37
Indigo carmin 70
Influence du pH dans les colorations par la méthode de Giemsa. (abs.) 32
Iris violet 74
Iron-aceto-carmin 53, 54
Iron hematoxylin 45, 61

Janus green 3, 74, 82
Jenner's stain 36
Jirouch, E. A. Natural color preserved in sectioned green plant tissue 17
Jordan, J. H. and A. H. Heather. Staining of Negri bodies 121
Joyet-Lavergne, Ph. La recherche qualitative du glutathion. (abs.) 95
Sur quelques procedes de recherche microscopique du glutathion dans les cellules. (abs.) 29

Kalkfärbung mit machfolgender Entkalkung, eine neue Methode. (abs.) 30
Kater, J. M. Cytology of *Saccharomyces cerviciae*. (abs.) 31
Kelley, A. P. and M. B. Shoemaker. Staining of yeast cells. (abs.) 32
Kisser, J, and B. B. Anderson. Method of preparing thin cross and longitudinal sections of cotton fibers and its importance in cell wall research. (abs.) 93

Lacorte, J. G. Influence du pH dans les colorations par la méthode de Giemsa. (abs.) 32
Lentze, Hrch. Anisol statt Zedernöl als Immersionsmittel nach Becher. (abs.) 133
Lepik, E. Differential staining of the Peronosporaceae. (abs.) 135
Levine, M. A method for staining connective tissue mast cells. (abs.) 95
Lewis, G. T.; F. P. Parker and. Microscopic projections in the measurements of erythrocytes. (abs.) 95
Light green SF yellowish 3, 27, 62, 66, 74
Lillie, R. D. Eine Schnellmethode zur Toluidinblau-Schleimfärbung. (abs.) 64
Kalkfärbung mit nachfolgender Entkalkung, eine neue Methode. (abs.) 30
Litmus 41
Loeffler's methylene blue, note on 27
Logwood dyes, staining with, history of 37
Logwood extract 37, 38, 39, 40, 41, 44, 46

Madder 37
Magdala red 121
Malachite green 113, 114

Maneval, W. E. Some staining methods for bacteria and yeasts 21
Martens, P. Les structures mucléaires et chromosomiques dans la cellule vivant et dans la cellule fixée. (abs.) 95
Martius yellow 74
Mascre, M. Sur la fixation de la chondriome de la cellule végétale. (abs.) 30
McClintock, Barbara. A method for making aceto-carmin smears permanent 53
Mechanism of staining. The case for the physical theories 75
Mechanism of staining explained on a chemical basis. I The reaction between dyes, proteins, and nucleic acid 111
Mercurochrome 135
Method for making aceto-carmin smears permanent 53
Method for staining connective tissue mast cells. (abs.) 95
Method for the study of chromosomes in pollen-mother-cells. (abs.) 94
Method of differential staining of the human brain. (abs.) 135
Method of preparing thin cross and longitudinal sections of cotton fibers and its importance in cell wall research. (abs.) 93
Method of staining bacterial flagella. (abs.) 31
Method of staining flagella. (abs.) 32
Methyl blue 23
Methyl green 23, 70, 78, 98, 102
Methyl-green-pyronin, Pappenheim-Unna's 102
Methyl orange 98
Methyl violet 24, 78, 95, 134
Methyl violet 2B 70
Methylene azure II 96
Methylene azure B 125
Methylene blue ... 7–10, 13, 24, 25, 27, 31, 35, 36, 49, 50, 51, 61, 63, 64, 81–85, 88, 94, 96, 98, 102, 107, 124, 126, 135
aqueous solutions of, atmospheric oxidation or dealkylation of 7
eosinate 52
Loeffler's, note on 27
medicinal 70, 81
polychrome 102
tablets 36
Methylene green 74
Methylene violet 7, 96
eosinate 52
Metodo Petragnani per le ciglia batteriche e le sue applicazionialla colorazione de tessuti animali. (abs.) 133
Microscopic projections in the measurements of erythrocytes. (abs.) 95
Millot, J. and Giberton, A. Sur utilization, en histologie, des pièces conservées dans le formol, pour la mise en évidence des graisses. (abs.) 30
Mitochondria and bacteria, double staining of in plant tissues 13
Mode de formation e la detection des alcaloides dans la plantule de tabac. (abs.) 61
Modification of Mayer's hemalum 127
Modification rapide de la méthode de Weigert-Pal. (abs.) 62
Molybdic hematoxylin 123

Nan, Yao. Sur un fixateur cytologique particulièrement adapté aux tissues des insectes. (abs.) 61
Natural color preserved in sectioned green plant tissue 13
Nature della coloration histologique et sur la methode de contact. (abs.) 136
Negri bodies, staining of 121
Neutral red 3, 61, 62, 74
Neutral violet 74
New staining dish for handling coverglass preparations 57
New victoria blue R 74
Nigrosin 3
Nile blue 2 B 74
Note on the Gram stain 91
Note on preparing muci-carmine. (abs.) 32
Notes on technic:
Differentiation of safranin in cytological work 27
Note on Loeffler's methylene blue 27

Nouvelle coloration élective de la myeline. (abs.) ... 64
Nouvelle modification de la technique d'Altman. (abs.) ... 96
Nuclear and differential stain combined. (abs.) ... 133
Nucleic acid, dyes, proteins and, reaction between ... 111
Nuovo metodo di colorazione dei grassi. (abs.) ... 94

Ochs, Georg Wilhelm. Über den Einfluss der Temperatur auf die Färbung von Blutausstrich-Präparaten. (abs.) ... 64
Orange G ... 31, 32, 63, 66, 95, 133, 135
Oxamine blue 4BX ... 135

Paraffin method for serially sectioning a minute object in a known plane. (abs.) ... 93
Paraffineinbettungsmethode nach vorhergegangener Zelloidindurchtränkung unter Vermeidung der härtenden Intermedien Xylol, Benzol, Chloroform. (abs.) ... 61
Parker, F. P. A technic for staining red blood cells for measurement of cell diameters by the projection method. (abs.) ... 95
Parker, F. P. and G. T. Lewis. Microscopic projections in the measurements of erythrocytes. (abs.) ... 95
Patent blue A ... 74
Pergola, M. Substrati al tellurito e colorazione ridotta nella ricerca e dimostrazione del bacillo difterico. (abs.) ... 96
Phloxine ... 70, 73, 74, 98, 121, 124, 125
Phloxine-eosin stain for Negri bodies ... 121, 124, 125
Picric acid ... 12, 29, 30, 32, 95, 123
Plant tissues, double staining of mitochondria and bacteria in ... 13
Plant tissue, green, natural color preserved in sections ... 17
Plant tissues, short method of clearing for anatomical studies ... 131
Ponceau 2 G ... 74
Praktische Winke sur Azur II-Eosinfärbung an Schnittpräparaten. (abs.) ... 136
Present members of the Commission on Standardization of Biological Stains ... 5
Procédé rapide de coloration des bacilles de Koch dans les coupes histologiques. (abs.) ... 94
Progress in the standardization of stains:
  Definiteness in staining formulae ... 33
  Hematein and acid fuchsin ... 97
  Influence of impurities upon dyes ... 68
  Safranin ... 65
  Stain specifications ... 1
  Stains recently certified ... 2, 36, 70, 98
Proteins, dyes, and nucleic acid, reaction between ... 111
Pyronin ... 3
Pyronin G ... 74

Rapid stain for direct microscopic examination of milk. (abs.) ... 61
Recherche qualitative du glutathion. (abs.) ... 95
Red cabbage ... 41
Reichardt, H. and A. Wetzel. Paraffineinbettungsmethode nach vorhergegangener Zelloidindurchtränkung unter Vermeidung der härtenden Intermedien Xylol, Benzol, Chloroform. (abs.) ... 61
Resorcin-fuchsin ... 11, 31
Résultats obtenus chez la seiche par l'emploi de divers colorants vitaux. (abs.) ... 62
Rhodamine B ... 63
Rhodamine G ... 74
Rosanilin ... 107
Rose bengal ... 23, 125
Rubin S ... 96

Safranin ... 23, 24, 27, 82, 95, 108, 134, 135
  Differentiation of, in cytological work ... 27
  Flemming ... 65, 66
  nach Curtis ... 65, 66
  rein (Phenosafranin) ... 65, 66
  spiritusloslich ... 65
  standardization of ... 65
  yellow, water soluble ... 65, 66

Safranin O 65–69
wasserloslich, Pfitzner 65
Sass, John E. Modification of Mayer's hemalum 127
Satisfactory method of staining fungal mycelium in wood sections. (abs.) 94
Schnellmethode zur Toluidinblau-Schleimfarbung. (abs.) 64
Shoemaker, M. B.; A. P. Kelley, and. Staining of yeast cells. (abs.) 32
Short method of clearing plant tissues for anatomical studies 131
Simpson, Jennie L. S. Short method of clearing plant tissues for anatomical studies 131
Smear method for the study of chromosomes. (abs.) 96
Snyder, E. F.; W. C. Holmes, and. The atmospheric oxidation, or dealkylation, of aqueous solutions of methylene blue 7
Southgate, H. W. Note on preparing muci-carmine. (abs.) 32
Spore stain, Dorner's 97
Stain specifications 1
Stain solubilities. Part IV 73
Staining, elastic tissue 11
History of: Logwood dyes 37
Mechanism of 75
Staining character of bacteria, variation, as related to reserve food material within the organism 105
Staining dish, new, for handling cover-glass preparations 57
Staining formulae, definiteness in 33
Staining mechanism explained on chemical basis. I. The reaction between dyes, proteins, and nucleic acid 111
Staining methods for bacteria and yeasts, some 21
Staining of yeast cells. (abs.) 32
Staining of Negri bodies 121
Stains recently certified 2, 36, 70, 98
Standardization of stains, progress in:
Definiteness in staining formula 33
Stain specifications 1
Hematein and acid fuchsin 97
Stearn, Allen E. and Esther W. Mechanism of staining explained on a chemical basis. I. The reaction between dyes, proteins, and nucleic acid 111
Stearn, Esther W. and Allen E. Stearn. Variation in staining character of bacteria as related to the reserve food material within the organism 105
Stegall, P. A. A method of differential staining of the human brain. (abs.) 135
Structures nucléaires et chromosomiques dans la cellule vivante et dans la cellule fixée. (abs.) 95
Studies in stain technique. I. Flemming's safranin, gentian violet, orange G method, with a suggested modification. (abs.) 135
Substrati al tellurito e colorazione ridotta nella ricerca e dimostrazione del bacillo difterico. (abs.) 96
Sudan 94
Sur la detection histochimique de l'adrenaline. (abs.) 29
Sur la fixation de la chondriome de la cellule végétale. (abs.) 30
Sur quelques procédés de recherche microscopique du glutathion dans les cellules. (abs.) 29
Sur utilization en histologie, des pièces conservées dans le formol, pour la mise en évidence des graisses. (abs.) 30
Sweany, H. C. System of sputum analysis for the presence of acid-fast bacilli. (abs.) 93
System of sputum analysis for the presence of acid-fast bacilli. (abs.) 93

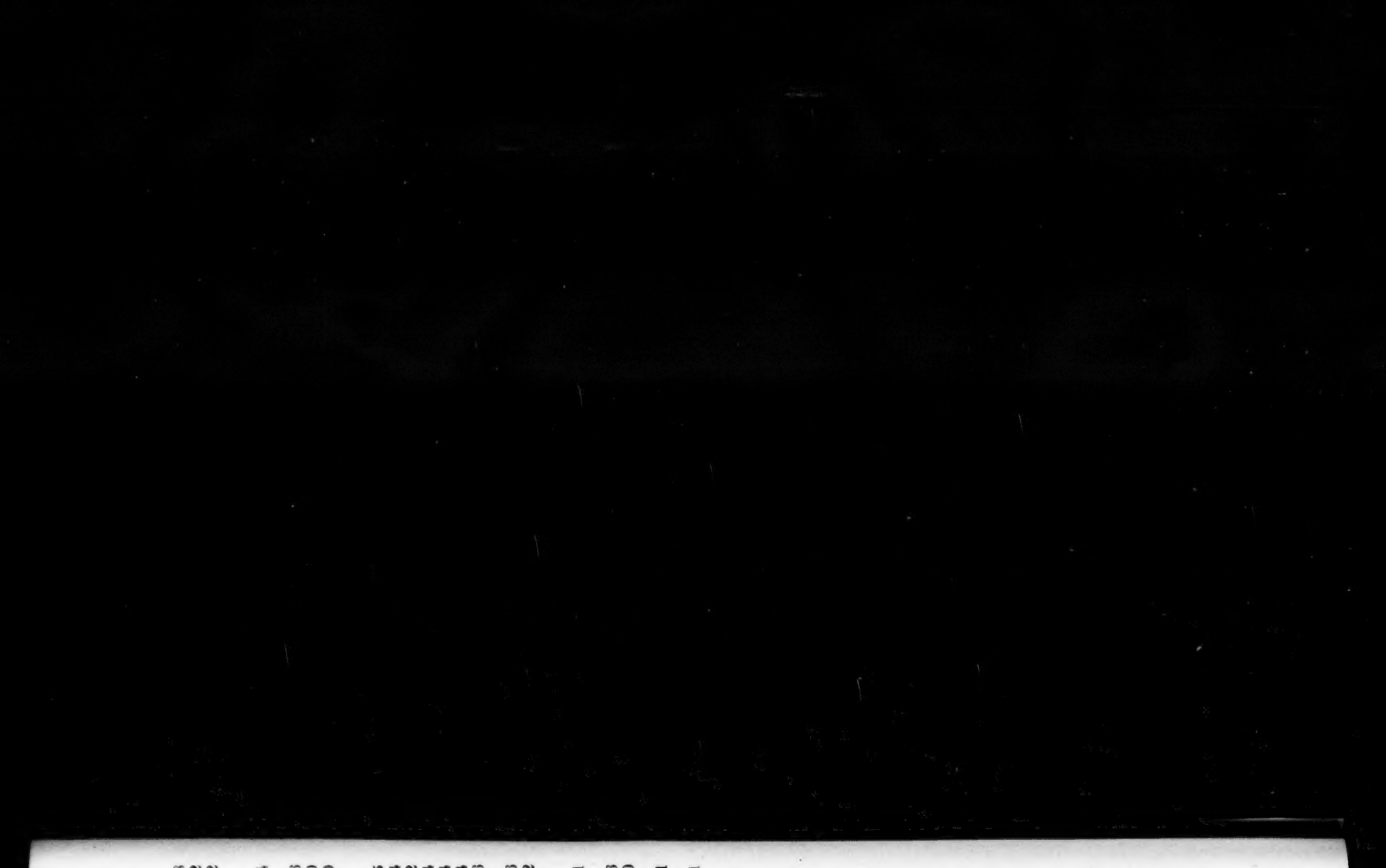
Technic for staining red blood cells for measurement of cell diameters by the projection method. (abs.) 95
Thiazin eosinates 49, 50, 52
Chemical analysis of 49
Thionin 63, 70, 95
Toluidin blue 63, 64
Tolstoouhov, Alexander V. Detailed differentiation of bacteria by means of a mixture of acid and basic dyes at different pH-values 81

Trimethyl thionin 7, 8
eosinate 52
Trypan blue 62

Über den Einfluss der Temperatur auf die Färbung von Blutausstrich-Präparaten. (abs.) 64
Ugriumow, B. Praktische Winke sur Azur II-Eosin-färbung an Schnittpräparaten. (abs.) 136
Unna, Eugen. Professor P. G. Unna 100
Unna, Professor P. G 100

Variation in staining character of bacteria as related to reserve food material within the organism 105
Verne, Jean. Une nouvelle coloration élective de la myeline. (abs.) 64
Victoria blue 62
Victoria blue 4 R 74
Victoria green 3 B 74
Victoria yellow 74
Volkonsky, M. Sur une nouvelle modification de la technique d'Altman. (abs.) .. 96

Webber, J. M. Smear method for the study of chromosomes. (abs.) 96
Weiss, E. Method of staining flagella. (abs.) 32
Wetzel, A.; H. Reichardt and. Paraffineinbettungsmethode nach vorhergegangener Zelloidindurchtränkung unter Vermeidung der härtenden Intermedien Xylol, Benzol, Chloroform. (abs.) 61
Wolfe, Jean. Sur la nature della coloration histologique et sur la methode de contact. (abs.) 136
Wright's stain 3, 49, 70

Yeasts and bacteria, some staining methods for 21

Zeissig, Alexander. A note on the Gram stain 91